Correlation between Fecal Concentrations of Ciprofloxacin and Fecal Counts of Resistant Enterobacteriaceae in Piglets Treated with Ciprofloxacin: toward New Means To Control the Spread of Resistance?

Thu Thuy Nguyen, Elisabeth Chachaty, Clarisse Huy, Carole Cambier, Jean de Gunzburg, France Mentré, Antoine Andremont

Correlation between fecal concentrations of ciprofloxacin and fecal counts of resistant *Enterobacteriaceae* in piglets treated with ciprofloxacin: towards new means to control the spread of resistance?

Thu Thuy Nguyen,[1,2†] Elisabeth Chachaty,[3†§] Clarisse Huy,[4] Carole Cambier,[5] Jean de Gunzburg,[4] France Mentré[1,2,7] and Antoine Andremont[1,6,7]

[1]University Paris Diderot, Sorbonne Paris Cité, Paris, France; [2]INSERM, UMR 738, Paris, France ; [3]Institut Gustave-Roussy, Paris, France; [4]Da Volterra, Paris, France; [5]University of Liège, Liège, Belgium; [6]EA3964, "Emergence de la résistance bactérienne in vivo", Paris, France; [7]AP-HP, Hospital Bichat, Paris, France

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CIPROFLOXACIN LEVELS AND RESISTANT BACTERIA IN FECES

† The two authors contributed equally to the work.
§ Corresponding author. Adresse: Service de Microbiologie Médicale, Département de Biologie et de Pathologie Médicale, Institut Gustave-Roussy, 114 rue Edouard-Vaillant, 94805 Villejuif, Paris, France. Phone: 33 (0)1 42 11 52 34. Fax : 33 (0)1 42 11 44 01. E-mail: elisabeth.chachaty@igr.fr.
This work was supported by a grant from DaVolterra.

Abstract

We assessed in a piglet model the relationship between fecal ciprofloxacin concentrations and ciprofloxacin resistant *Enterobacteriaceae* counts. Twenty-nine piglets were orally treated with placebo, ciprofloxacin 1.5 or 15 mg/kg/day from day D1 to D5. Areas under the curve (AUC) of concentrations increased sharply with dose and correlated positively with AUC of resistant bacteria log counts between D1 and D9. Removing residual colonic quinolones could help to control the emergence of resistance in fecal flora.

Increasing resistance to fluoroquinolones in Gram-negative bacilli is of major concern because it compromises their therapeutic use (6, 7). Colonic flora is the reservoir of many of the *Enterobacteriaceae* species with clinical significance (2) which are exposed to antibiotic residues during fluoroquinolone treatments (9). It has been shown that administration of enrofloxacin, a fluoroquinolone used in animals, could promote the emergence of quinolone resistant enterobacteria in fecal flora of pigs (12). We also found that administration of oral ciprofloxacin is associated with the emergence of quinolone resistant strains of *Enterobacteriaceae* in fecal flora from human volunteers (3). However, rates of emergence of resistance were not significantly different although the volunteers were exposed to different antibiotic dosages. Of note, detection of resistance was only qualitative in that study, i.e. absence versus presence of detectable resistant bacteria, and not quantitative, expressing the densities of resistant bacteria in the feces. Indeed, several lines of evidence suggest that quantitative aspects of emergence of resistance should also be analyzed. For instance, in neutropenic patients, Gram-negative bacilli bacteremias are caused by the predominant fecal clone of this type (11). Also, the density of a given *Escherichia coli* strain in a subject's fecal flora can influence to some extent its ability to cause urinary tract infection (8). In order to explore further the dynamics of fluoroquinolone resistance in the fecal flora during treatments, we assessed here in a prospective randomized study in piglets the relationship between fecal concentrations of ciprofloxacin and amounts of excreted ciprofloxacin resistant *Enterobacteriaceae*.

Twenty-nine piglets from a single farm were included 4 weeks after birth. They were born from sows that were treated at the time of parturition with 2 g oxytetracycline given intramuscularly, but had not received directly any antimicrobials. Piglets were housed in individual boxes for 21 days before the start of treatment (D1) and were randomly assigned to oral treatment with placebo (9 piglets), ciprofloxacin 1.5 mg/kg/day (10 piglets) or 15

mg/kg/day (10 piglets) from D1 to D5. Ciprofloxacin suspension (Ciflox®, Bayer) or mineral water (placebo group) was administered once a day at least 4 hours before food to animals fasted for at least 12 hours. The protocol was approved by the local ethical committee.

Fecal samples were recovered from piglets after anal stimulation on mornings before treatment (at D-1 and D1), during treatment (at D3 and D5, before ciprofloxacin administration), and after treatment (at D7 and D9). Samples were stored frozen at -80°C until microbiological analysis and ciprofloxacin assay were performed blinded of the treatment group. Fecal concentrations of ciprofloxacin were measured by microbiological assay (5) after ten-fold dilution in HCl 0.1N and then further dilutions in phosphate buffer pH 8.0. Counts of total and resistant *Enterobacteriaceae* were obtained after plating serial dilutions of fecal samples on Drigalski agar (BioRad laboratories, Marnes-la-Coquette, France) supplemented or not with 20 mg/l of nalidixic acid or with 2 mg/l of ciprofloxacin. However, since there was no significant differences in the counts obtained on the two types of media (data not shown), only counts on ciprofloxacin containing agar were used for further statistical analysis. Individual area under the curve (AUC) from D1 to D9 of ciprofloxacin fecal concentrations (AUC_CIP), of log counts (AUC_RES) and of percentages (AUC_%RES) of ciprofloxacin resistant *Enterobacteriaceae* above pre-treatment baseline values were computed by trapezoidal approach. These criteria were compared across groups by nonparametric ANOVA and pairwise Wilcoxon tests if global tests were statistically significant (with significance level at 0.05). All statistical analyses were performed using SAS 9.1 (SAS, Cary, NC).

Concentrations of ciprofloxacin increased with the dose administered and peaked at 84.8±57.9 *versus* 11.6±12.6 µg/g of feces at D5 in piglets treated with 15 and 1.5 mg/kg/day of ciprofloxacin respectively ($p<0.001$) (Fig. 1A). Fecal antibiotic activity was detectable to some extent in all animals 2 days after cessation of treatment (D7) but no activity was detected in any animal at D9. AUC_CIP was significantly higher for the group treated with 15

mg/kg/day of ciprofloxacin than that for the group treated with 1.5 mg/kg/day ($p<0.0005$) (Table 1).

Most of the piglets (25/29, 86%) had ciprofloxacin resistant *Enterobacteriaceae* detected in fecal samples before treatment. Administration of ciprofloxacin promoted the increase of both absolute counts (Fig. 1B) and percentages of ciprofloxacin resistant enterobacteria (Fig. 1C) in the 2 treatment groups when compared with the placebo group. AUC_RES and AUC_%RES were significantly different between the groups treated with 1.5 mg/kg/day and 15 mg/kg/day *versus* the placebo group (both $p<0.01$ for AUC_RES and both $p<0.0005$ for AUC_%RES). AUC_RES and AUC_%RES were also significantly higher for the group treated with 15 mg/kg/day than that for the group treated with 1.5 mg/kg/day ($p<0.05$ and $p<0.001$ respectively) (Table 1).

There were significant correlations between AUC_CIP and AUC_RES as well as between AUC_CIP and AUC_%RES (both $p<0.0001$, Spearman tests). These relationships were adequately described using an Emax model: $AUC\_RES = AUC\_RES_0 + \frac{AUC\_RES_{max} \times AUC\_CIP}{AUC\_CIP_{50} + AUC\_CIP}$ where AUC_RES_0, AUC_RES_{max} and AUC_CIP_{50} are respectively the baseline, the maximal effect and the value of AUC_CIP to obtain 50% of the maximal effect (Fig. 2).

We showed a significant positive correlation between the AUC of fecal ciprofloxacin concentrations and the AUC of log ciprofloxacin resistant *Enterobacteriaceae* counts from D1 of treatment to D9 post treatment. Rapid increase of ciprofloxacin resistant enterobacteria from the beginning of ciprofloxacin administration probably resulted from the selection of resistant bacteria which were already present in low counts before treatment in the fecal flora of most (86%) of our piglets. Indeed, in a previous study in human volunteers (3), only 6 of 48 (12.5%) had quinolone resistant enterobacteria detected before treatment and the increased

prevalence of resistance was only observed after the end of the treatment without significant dose relationship. The piglet study described here was much more fitted to evidence pharmacokinetic/pharmacodynamic relationship since the doses varied by a factor of 10, starting from a very low dose to a clinical dose (from 1.5 to 15 mg/kg/day) whereas they only varied by a factor of 3 (250 mg twice/day to 750 mg twice/day) in the volunteers. Median ciprofloxacin fecal concentrations at steady state were also much lower in piglet than in human volunteers groups of treatment (7 and 73 versus 845 and 1938 µg/g of feces respectively). An increase in the percentage of ciprofloxacin resistant *Bacteroides fragilis* strains in feces of human-flora-associated mice treated with various doses of ciprofloxacin has been also reported as dose-related (10). However, our results differ from the absence of correlation between antibiotic dosage and the percentages of fecal quinolone resistant enterobacteria during treatment that was observed by others in pigs receiving several doses of enrofloxacin varying in a range of 1 to 6 (12). Dynamic of emergence of resistant bacteria is dependant of several parameters among which the concentrations of free and active antibiotic reaching the colonic flora, MICs of bacterial populations and barrier effects exerted in the colonic ecosystem. This may account for the nonlinear correlation between fecal antibiotic concentrations and level of resistant bacteria we observed and, also, for differences in results from similar studies.

We used AUCs to characterize the fecal pharmacokinetic/pharmacodynamic relationship for ciprofloxacin, taking into consideration the whole time-course of concentrations and bacteria counts. We believe that AUCs of fecal counts are the most relevant end-points because they describe, better than single time point values, the total amount of resistant bacteria excreted, which is actually what one wants to decrease to control the spread of resistance.

Indeed, the link we showed between intestinal concentration of ciprofloxacin and the amount of ciprofloxacin-resistant enterobacteria excreted by the animals may be of upmost

importance in the current context of the spread of bacterial resistance. It has been shown in a rat model that the administration of a compound consisting of activated charcoal entrapped within zinc-pectinate beads could remove residual colonic ciprofloxacin (4). Thus, if such a removal could be obtained in animals or humans receiving therapeutic doses of quinolones without impairing the pharmacokinetics of the drug in plasma, this would open new avenues within the scope of the recently signaled eco-evo drugs (1) to help to control of the emergence and spread of quinolone resistance in gut-originating Gram-negative bacteria.

This work was conducted in part with a grant from Da Volterra and the authors would like to thank the Professor Pascal Gustin, Service of Pharmacology, University of Liège, Belgium for his scientific advices.

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169 TABLE 1. Descriptive statistics by treatment group on AUC of ciprofloxacin concentrations
170 (AUC_CIP), AUC of log counts of ciprofloxacin resistant *Enterobacteriaceae* above baseline
171 (AUC_RES)[a] and AUC of percentages of *Enterobacteriaceae* resistant to ciprofloxacin
172 (AUC_%RES).

Treatment group	Mean±SD Median (Range)		
	AUC_CIP (μg×day/g)	AUC_RES (log CFU×day/g)	AUC_%RES (%×day)
Placebo (n=9)	0 0	-1.3±5.3 0.5 (-13.5-5.1)	4.5±6.1 0.9 (0.1-15.6)
Ciprofloxacin 1.5mg/kg/day (n=10)	44.0±32.2 30.6 (10.1-113.5)	7.6±6.2 7.1 (-0.2-21.5)	284.9±124.8 261.8 (86.5-504.8)
Ciprofloxacin 15mg/kg/day (n=10)	362.5±179.8 299.3 (191.2-711.5)	14.7±7.6 15.0 (-1.0-26.0)	567.1±98.2 568.0 (403.9-700.0)

173 [a] Some values of AUC_RES are negative because of the normalisation by the baseline.

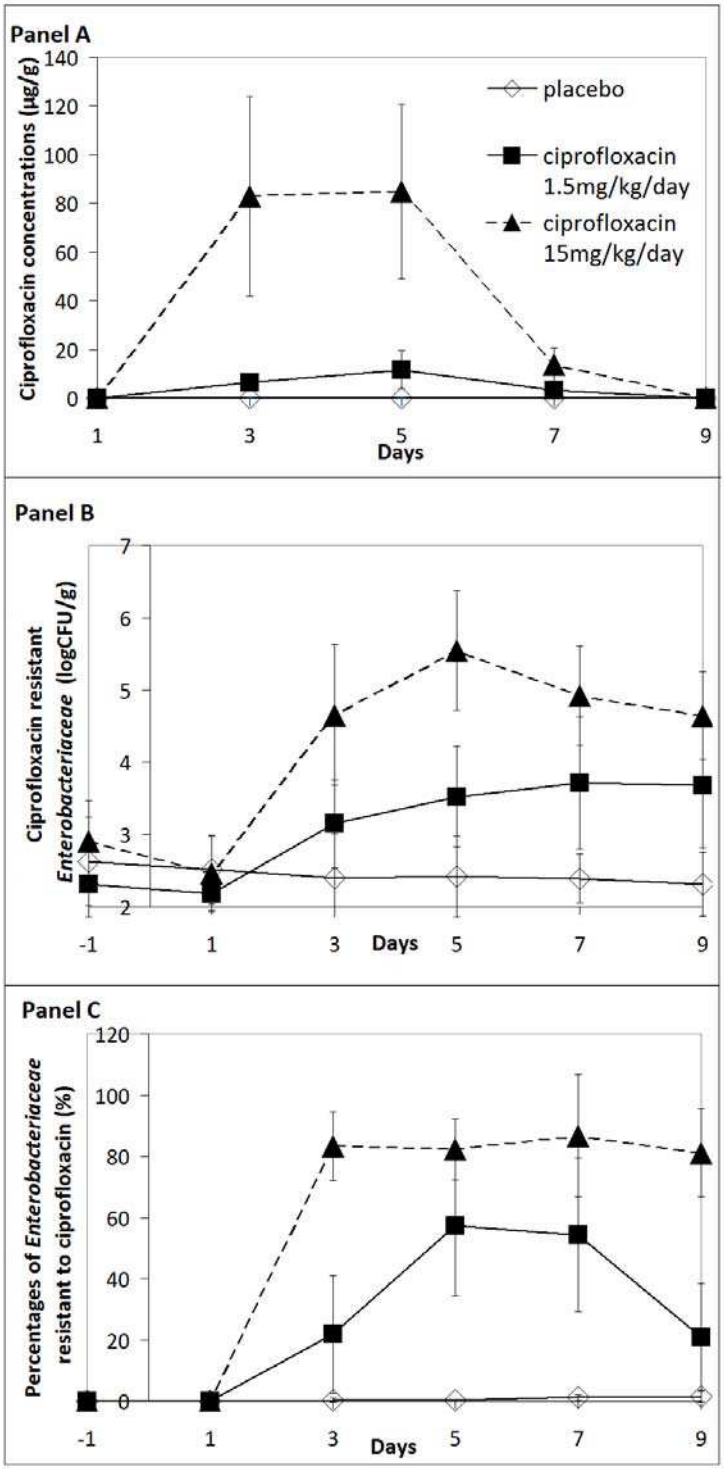

FIG. 1. Mean (and 95% confidence intervals) of ciprofloxacin concentrations (Panel A) and of log counts of ciprofloxacin resistant *Enterobacteriaceae* (Panel B) as well as of percentages of *Enterobacteriaceae* resistant to ciprofloxacin (Panel C) in fecal samples from piglets treated with placebo (n = 9), 1.5 mg/kg/day (n = 10) and 15 mg/kg/day (n = 10) of oral ciprofloxacin from day 1 to day 9.

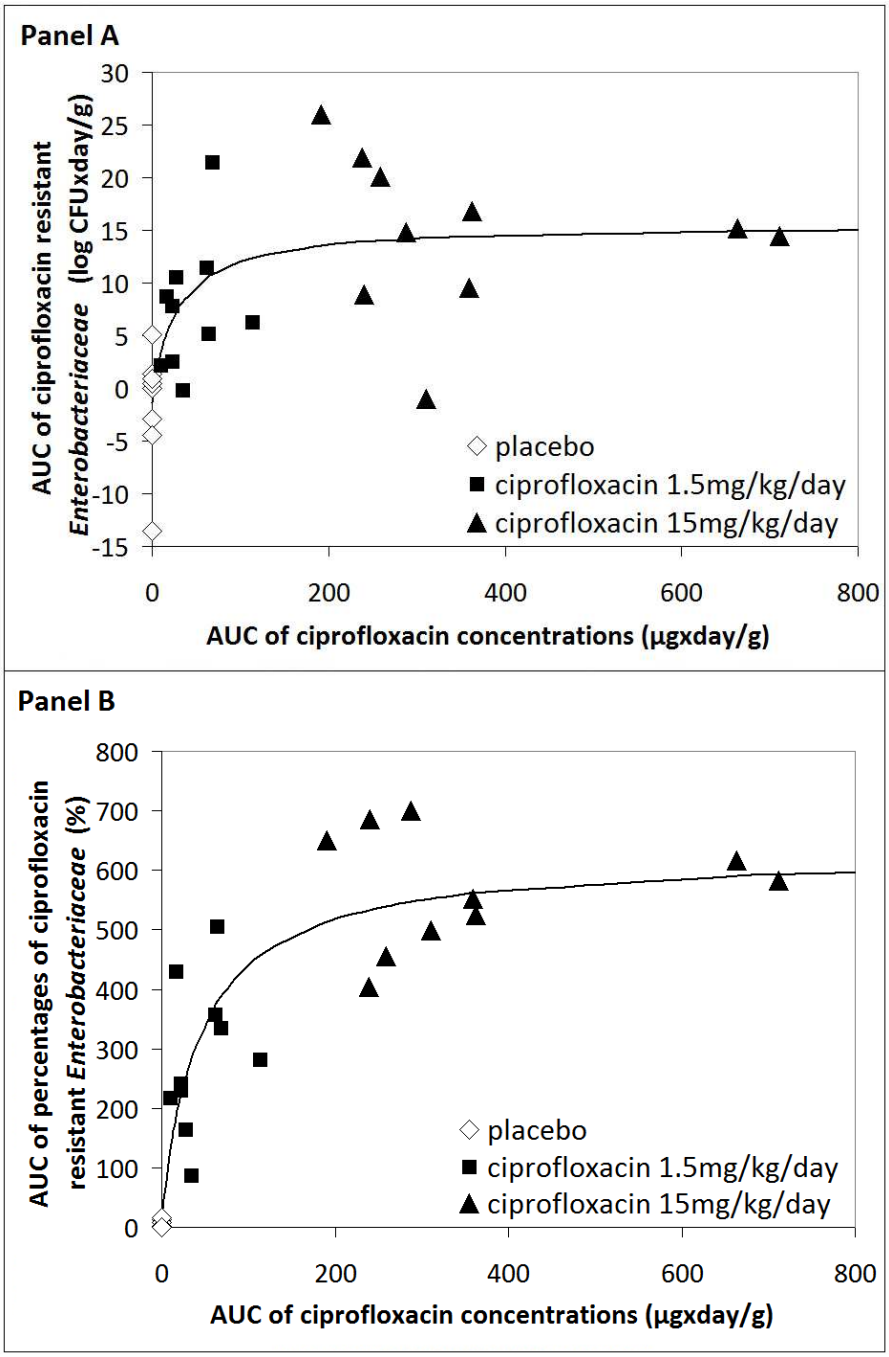

FIG. 2. Relationship between individual AUC of fecal concentrations of ciprofloxacin (AUC_CIP) *versus* AUC of log counts of fecal ciprofloxacin resistant *Enterobacteriacae* (AUC_RES) (Panel A) and *versus* AUC of percentages of ciprofloxacin resistant *Enterobacteriacae* (AUC_%RES) (Panel B) for 29 piglets in 3 treatment groups. Each symbol represents the AUC_CIP and AUC_RES or AUC_%RES values for one piglet. The black curve among the symbols is the mean curve predicted by the Emax model.